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sox2 and sox3 play unique roles in development of hair cells and neurons in the zebrafish inner ear

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Abstract

Formation of neural and sensory progenitors in the inner ear requires *Sox2* in mammals, and in other species is thought to rely on both *Sox2* and *Sox3*. How *Sox2* and/or *Sox3* promote different fates is poorly understood. Our mutant analysis in zebrafish showed that *sox2* is uniquely required for sensory development while *sox3* is uniquely required for neurogenesis. Moderate misexpression of *sox2* during placodal stages led to development of otic vesicles with expanded sensory and reduced neurogenic domains. However, high-level misexpression of *sox2* or *sox3* expanded both sensory and neurogenic domains to fill the medial and lateral halves of the otic vesicle, respectively. Disruption of medial factor *pax2a* eliminated the ability of *sox2/3* misexpression to expand sensory but not neurogenic domains. Additionally, mild misexpression of *fgf8* during placodal development was sufficient to specifically expand the zone of prosensory competence. Later, cross-repression between *atoh1a* and *neurog1* helps maintain the sensory-neural boundary, but unlike mouse this does not require Notch activity. Together, these data show that *sox2* and *sox3* exhibit intrinsic differences in promoting sensory vs. neural competence, but at high levels these factors can mimic each other to enhance both states. Regional cofactors like *pax2a* and *fgf8* also modify *sox2/3* functions.

Keywords

SoxB1; sensory epithelia; statoacoustic ganglion; otic placode

INTRODUCTION

Development of the inner ear is a highly dynamic process in which neurons, sensory epithelia, and a variety of non-sensory cell types arise from a simple epithelial structure, the otic placode. The otic placode quickly forms a fluid-filled cyst, the otic vesicle, which then further elaborates the complex shape and array of cell types comprising the inner ear. Sensory epithelia and neural progenitors originate in a ventral region of the early otic

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vesicle. In mammals and birds, neuroblasts arise first and subsequently delaminate from the otic epithelium and are quickly replaced by developing sensory epithelia (Raft and Groves, 2015; Raft et al., 2007). In zebrafish, neuroblasts and sensory epithelia initially form simultaneously in abutting domains in the floor of the otic vesicle, after which neuroblasts delaminate and differentiate in a manner similar to tetrapod vertebrates (Haddon and Lewis, 1996; Kantarci et al., 2016; Millimaki et al., 2007). Despite differences in timing and degree of spatial overlap of neural vs. sensory development, many of the same regulatory genes operate in all vertebrate species. The transition from neural to sensory development in mammals is triggered in part by cross-repression between proneural and prosensory factors *Ngn1* and *Atoh1* (Raft et al., 2007). In principle a similar cross-repression could help stabilize spatial segregation of sensory and neural fates in zebrafish, although this has not been formally investigated.

In all vertebrates, members of the SoxB1 family of transcription factors are required for normal development of both sensory epithelia and neurons of the inner ear (reviewed by Raft and Groves, 2015). In non-mammalian vertebrate species, *Sox3* is the first to be expressed during placodal development and appears to presage neural development, marked by expression of the proneural gene *Neurog1* (Abello et al., 2010; Neves et al., 2007; Nikaido et al., 2007; Sun et al., 2007). *Sox2* is expressed at later stages and is associated with development of sensory epithelia, marked by expression of the prosensory gene *Atoh1* (Millimaki et al., 2010; Neves et al., 2007). However, *Sox2* and *Sox3* show overlapping domains of expression for extended periods of otic development. This raises questions about whether *Sox2* and *Sox3* act redundantly in otic development, and how their cell-type specific functions are regulated (i.e. how do they differentially activate *Neurog1* vs. *Atoh1*). In mammals, *Sox3* is not expressed in the otic vesicle, whereas *Sox2* is expressed in the floor of the otic vesicle and is required for both neurons and sensory epithelia (Kiernan et al., 2005; Puligilla et al., 2010; Steevens et al., 2017). Moreover, replacement of the *Sox3* coding region with *Sox2* permits development of mice that are viable and morphologically normal, suggesting that *Sox2* and *Sox3* are largely redundant (Adikusuma et al., 2017). Nevertheless, it is still not understood how *Sox2* can regulate both neurons and sensory epithelia in the same tissue, a problem that is presumably related to how *Sox2* and *Sox3* regulate different cell fates in non-mammalian vertebrates.

SoxB1 factors are well known for providing two seemingly contradictory functions. They can maintain pluripotency of stem cells and progenitors (Bylund et al., 2003; Goldsmith et al., 2016; Rizzino and Wuebben, 2016; Surzenko et al., 2013; Tucker et al., 2010), and they can promote early differentiation of various cell types and tissues (Amador-Arjona et al., 2015; Archer et al., 2011; Hoffmann et al., 2014; Okuda et al., 2010; Rogers et al., 2009). A number of studies have shown that the nature of SoxB1 function can vary depending on their level of expression (Boer et al., 2007; Hutton and Pevny, 2011; Kopp et al., 2008; Rizzino and Wuebben, 2016) or the availability of region- or stage-specific cofactors (Ambrosetti et al., 1997; Boer et al., 2007; Chew et al., 2005; Kamachi et al., 2001; Kondoh and Kamachi, 2010). It is unknown which of these variables regulates the ability of Sox2 and Sox3 (or Sox2 alone) to differentially regulate neural and sensory fates in the otic vesicle.

We have investigated the shared and unique functions of *sox2* and *sox3* in zebrafish through analysis of knockout lines and heat shock-inducible transgenes. Mutant analysis confirms that *sox2* is uniquely required for normal sensory development whereas *sox3* is uniquely required for neural development. Some misexpression studies also support these gene-specific functions. For example, misexpression of *sox2* at a moderate level during placodal development expands the domain of sensory development in the otic vesicle while restricting the domain of neurogenesis. However, when misexpressed at high levels, *sox2* and *sox3* mimic each other and lead to dramatic expansion of sensory and neural fates throughout the medial and lateral walls of the otic vesicle, respectively. The ability to expand sensory fates, but not neural fate, requires the medial factor *pax2a*. Early misexpression of *sox2* or *sox3* did not accelerate the onset of sensory or neural development, rather expansion of these fates occurred gradually after formation of the otic vesicle. Moreover, misexpression of *sox2* or *sox3* at later stages temporarily halts expression of prosensory and proneural factors *atoh1a* and *neurog1*. Analysis of additional markers shows that misexpression of *sox2* or *sox3* expands anterior-ventral identity (including the zones of sensory-neural competence) throughout the otic vesicle. Together these data suggest that *sox2* and *sox3* promote sensory-neural competence while delaying onset of sensory and neural differentiation. Regionally expressed factors such as *pax2a* and *fgf8* then help to diversify *sox2/3* function to establish sensory and neural fates in spatially segregated domains. We also confirm that cross-repression between *atoh1a* and *neurog1* help reinforce the sensory-neural boundary.

MATERIALS AND METHODS

Fish strains and developmental conditions

Wild-type zebrafish were derived from the AB line (Eugene, OR). Mutant alleles *sox2*^{x50}, *sox3*^{x52} (Gou et al., 2017) and *mib1a*^{52b} (Itoh et al., 2003; Jiang et al., 1996) and genotyping methods were previously described. Transgenic line *TG(brn3c:gap43-GFP)* (Xiao et al., 2005) was used to visualize sensory hair cells. Transgenic lines *TG(hsp70:fgf8a)*^{x17}, *TG(hsp70:atoh1a)*^{x20}, *TG(hsp70:sox2)*^{x21} (Millimaki et al., 2010), *TG(hsp70:sox3)*^{x32} (Gou et al., 2017) and *TG(hsp70:ngn1)*^{x28} (Kantarci et al., 2016) used in the misexpression studies here were referred to as *hs:fgf8*, *hs:atoh1a*, *hs:sox2*, *hs:sox3* and *hs:neurog1* respectively. Embryos were developed under standard conditions at 28.5°C (Kimmel et al., 1995), except during and after heat shock, in fish water containing methylene blue. Embryos were staged based on standard morphological features (Kimmel et al., 1995). To prevent melanin formation in older embryos (>24 hpf), PTU (1-phenyl 2-thiourea, 0.3 mg/ml) was added to fish water during development.

Gene misexpression and morpholino injections

Misexpression of various genes was achieved by briefly incubating embryos heterozygous or homozygous for heat shock inducible transgenes in a water bath at 38 or 39°C for 30 or 60 minutes, except where noted in the text. For experiments involving activation of *hs:sox3* at 12 hpf, heat shock was performed at 38°C because 39°C heat shock at this time led to severe axial truncation by 24 hpf, precluding meaningful interpretation of results. In contrast, activation of *hs:sox3* at 39°C was readily tolerated at later stages. In all cases, after heat-shock embryos were maintained at 33°C until fixation. Wild-type embryos were also heat

shocked to serve as controls for all misexpression studies. Knock-down of *pax2a* was achieved by injecting 5 ng of morpholino oligomers (mo), obtained from Gene Tools, Inc, into one-cell stage embryos. Sequence of *pax2a*-mo was previously described (Bricaud and Collazo, 2006). In all morpholino knock-downs, embryos were co-injected with *p53*-mo (Robu et al., 2007) to prevent non-specific cell death. Phenotypes described here were assessed in at least 15 embryos per probe and time point unless stated otherwise.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization, two-color in situ hybridization and immunostaining were performed as previously described (Jowett and Yan, 1996; Phillips et al., 2001; Riley et al., 1999). Primary antibody used to label mature neurons of statoacoustic ganglion (SAG) is anti-Islet1/2 (Developmental Studies Hybridoma Bank 39.4D5, 1:100), secondary antibody is Alexa 546 goat anti-mouse IgG (ThermoFisher Scientific A-11003, 1:50). For cell proliferation analysis, anti-phospho-Histone H3 (EMD MILLIPORE 06-570, 1:350) and Alexa 546 goat anti-rabbit IgG (ThermoFisher Scientific A-11010, 1:50) were used.

Cell transplantation and cryo-sectioning

Donor *hs:sox3/hs:sox3* embryos were injected with lineage tracer (10,000 MW, lysine-fixable tetramethylrhodamine labeled dextran in 0.2 M KCl) at one-cell stage. Donor cells were transplanted into unlabeled wild-type host embryos during late blastula stage. Embryos stained by whole-mount in situ hybridization were processed for cryo-sectioning as previously described (Vemaraju et al., 2012) and cut into serial 10- μ m sections then mounted in 30% glycerol, except that sections from mosaic embryos were mounted in SlowFade Gold antifade reagent with DAPI (Life technologies).

Genotyping and data analysis

To identify *sox2*^{-/-}, *sox3*^{-/-} single mutants and *sox2*^{-/-}; *sox3*^{-/-} double mutants from *sox2*^{+/-}; *sox3*^{+/-}; *brn3c:gap43-GFP* triple carrier intercross, tails of individual embryos, post Islet1/2 immunostaining for Fig. 2B, were used for DNA extraction and single-embryo genotyping described previously (Gou et al., 2017). Quantification of gene expression area was performed using Photoshop measuring number of pixels. Areas shown in the figures were normalized relative to wild-type control embryos. Quantification of number of cells expressing certain gene or protein was done in either whole mounts (*atoh1b*, phospho-Histone H3 and Islet1/2 staining) or in serial sections (*atoh1a*, *neurog1* staining in mosaic embryos). Hair cells were counted in fixed whole mount embryos by imaging *TG(brn3c:gap43-GFP)* fluorescence, a stable marker of mature hair cells (Xiao et al., 2005). Mature SAG neurons were counted in whole mounts stained with anti-Islet1/2 monoclonal antibody. Expression of Islet1/2 marks SAG cells that have completed migration, become post-mitotic, and sprouted projections to synaptic targets (Vemaraju et al., 2012). Statistical pair-wise comparisons were performed using students' t-test. For experiments that involve more than two groups, significance was evaluated by ANOVA and Tukey's post-hoc HSD tests.

RESULTS

Expression of *sox3* in the otic vesicle

Although expression of *sox3* is a well known marker of early development of the otic placode (Nikaido et al., 2007; Sun et al., 2007), expression in the otic vesicle has not been described. We therefore characterized expression of *sox3* in the otic vesicle at 24 hpf with respect to *neurog1* in the neurogenic domain, *atoh1a* in sensory maculae, and the medial marker *pax2a* (Fig. 1A). Cross sections through the utricular macula show that *sox3* is expressed at a relatively low level and overlaps with both *atoh1a* and *pax2a* (Fig. 1B-D). Slightly more posterior sections passing through the widest part of the neurogenic domain show that *sox3* overlaps extensively with *neurog1*, but neither gene overlaps with *pax2a* (Fig. 1E-G). Additionally, *sox3* is expressed in a gradient, with high levels in the medial half of the neurogenic domain and falling rapidly towards the lateral half. The lateral domain corresponds to the region where neuroblasts delaminate, a function that requires overlap of *neurog1* with a lateral domain of *gooseoid* expression (Kantarci et al., 2016). Thus, *sox3* is widely expressed in the floor of the otic vesicle but shows highest expression in newly specified neuroblasts, with levels declining as neuroblasts mature and delaminate. Additionally, the sensory-neural boundary corresponds closely to the *pax2a* expression domain. We previously reported that *sox2* expression is restricted to prosensory regions of the otic placode and vesicle (Millimaki et al., 2010) and that sensory epithelia coexpress and upregulate *pax2a* (Riley et al., 1999).

Requirements for *sox2* and *sox3* in sensory and neural development

To test whether *sox2* and *sox3* play redundant or distinct roles in otic development, we examined accumulation of hair cells and neurons of the statoacoustic ganglion (SAG) in embryos disrupted for *sox2* or *sox3* or both *sox2* and *sox3*. *sox2*^{-/-} mutants show a 25% reduction in the number of hair cells at 38.5 hpf (Fig. 2A) and often display 1-2 dying hair cells being extruded from the otic epithelium, similar to previous findings in *sox2* morphants (Millimaki et al., 2010). In contrast, the number of mature SAG neurons at 36 hpf is normal in *sox2*^{-/-} mutants (Fig. 2B). Conversely, *sox3*^{-/-} mutants produce a normal number of hair cells (Fig. 2A) but the number of mature SAG neurons is reduced by 23% at 36 hpf (Fig. 2B). Interestingly, *sox2*^{-/-};*sox3*^{-/-} double mutants do not show additional reduction in accumulation of hair cells or SAG neurons compared to *sox2*^{-/-} and *sox3*^{-/-} single mutants, respectively (Fig. 2A, B). These data show that *sox2* and *sox3* are uniquely required for sensory and neural development, respectively, and there is no synergistic interaction between *sox2* and *sox3*.

Misexpression of *sox2* and *sox3*.

To further explore the unique and overlapping functions of *sox2* and *sox3*, we tested the effects of misexpressing *sox2* or *sox3* at different developmental stages and with varied expression levels using heat shock-inducible transgenes. We began by misexpressing *sox2* or *sox3* at 12.5 hpf (7 somites stage), when developing otic cells are still uncommitted. For moderate misexpression, transgenic *hs:sox2*⁺ and *hs:sox3*⁺ heterozygotes were heat shocked for 30 minutes at 38-39°C (see Materials & Methods), yielding a pulse of transgene activity lasting roughly 2-3 hours after the end of the heat shock period (Padanad et al.,

2012). Moderate misexpression of *sox2* at 12.5 hpf led to a modest expansion of sensory epithelia at 24 hpf as shown by a slightly expanded domain of *atoh1a* (Fig. 2D) and a 29% increase in mature hair cells by 30 hpf (Fig. 2M). Under these same conditions, there was a marked reduction in the neurogenic domain at 24 hpf marked by *neurog1* (Fig. 2G) and the number of mature SAG neuron was reduced by 21% at 30 hpf (Fig. 2N). In contrast to *sox2*, moderate misexpression of *sox3* expanded both sensory and neurogenic domains at 24 hpf (Fig. 2E, H). Sections through the middle of the otic vesicle revealed ectopic *atoh1a* expressing cells in the medial wall of the otic vesicle (Fig. 2J compare to 2I), while ectopic *neurog1* expressing cells appeared in the lateral wall of the otic vesicle (Fig. 2L compare to 2K). At later stages there was a corresponding increase in the number of mature hair cells and SAG neurons (Fig. 2O, P). Thus, when misexpressed at moderate levels, *sox2* promotes sensory development and impairs neurogenesis, whereas *sox3* promotes both sensory and neural development.

To achieve higher levels of misexpression, we increased transgene copy-number by generating *hs:sox2/hs:sox2* and *hs:sox3/hs:sox3* homozygotes. High-level misexpression of *sox2* at 12.5 hpf led to a dramatic expansion of both sensory (Fig. 3B compare to 3A) and neurogenic domains by 24 hpf (Fig. 3F compare to 3E), with ectopic *atoh1a* expressing cells filling the medial wall (Fig. 3I) and some ectopic *neurog1* expressing cells spreading into the lateral wall (Fig. 3L). High-level misexpression of *sox3* at 12.5 hpf usually led to similar but even more pronounced expansion of sensory and neurogenic domains at 24 hpf (Fig. 3C, G), such that almost all medial cells express *atoh1a* and almost all lateral cells express *neurog1* (Fig. 3J, M). However, in a subset of *hs:sox3/hs:sox3* embryos, the neurogenic domain expanded to encompass nearly the entire otic vesicle including the medial wall (Fig. 3H, N) with a corresponding contraction of the sensory domain (Fig. 3D, K). Thus, when expressed at high levels, *sox2* and *sox3* can greatly expand both sensory and neural fates, and in extreme cases *sox3* can lead to acquisition of neural fate by nearly all otic cells.

We next tested the effects of misexpressing *sox2* or *sox3* at later stages of otic development. Moderate misexpression of *sox2* at 18 hpf caused no obvious changes in expression of *atoh1a* or *neurog1* at 24 hpf (data not shown), and high-level misexpression of *sox2* at 18 hpf caused only a modest increase in sensory and neurogenic domains at 24 hpf (Fig. 4D, H). Moderate misexpression of *sox3* at 18 hpf also led to a modest expansion of sensory and neurogenic domains at 24 hpf (Fig. 4B, F). In contrast, high-level misexpression of *sox3* at 18 hpf strongly reduced expression of *atoh1a* and *neurog1* at 24 hpf (Fig. 4C, G), indicating suppression of sensory and neural development.

Moderate misexpression of *sox2* or *sox3* at 24 hpf had no discernable effect on subsequent sensory or neurogenic domains (data not shown), but high-level misexpression at 24 hpf strongly suppressed expression of *atoh1a* and *neurog1* by 26 hpf (Fig. 4I-k, O-Q). By 30 hpf, expression of *atoh1a* and *neurog1* recovered to near normal in *hs:sox2/hs:sox2* embryos (Fig. 4M, S), whereas *hs:sox3/hs:sox3* embryos continued to show partial suppression of *atoh1a* and *neurog1* (Fig. 4N, T). Thus, the ability of *sox2* and *sox3* to expand sensory and neurogenic domains is gradually lost during later stages of otic development and instead high-level misexpression strongly suppresses sensory and neural development. This suggests that *sox2* and *sox3* promote an early state of sensory and neural competence while delaying

early fate-specification, similar to their roles in establishing the neural plate during gastrulation (Archer et al., 2011; Bylund et al., 2003; Okuda et al., 2010; Rogers et al., 2009).

A delayed response to early misexpression of *sox2* and *sox3*.

To better characterize the effects of early misexpression, we examined when changes in *atoh1a* and *neurog1* expression first become evident following high-level misexpression of *sox2* and *sox3*. In *hs:sox2/hs:sox2* embryos heat shocked at 12.5 hpf, expression of *atoh1a* was normal through 16.5 hpf (Fig. 5B), with the first signs of moderate expansion appearing by 18.5 hpf (Fig. 5E). Expression of *neurog1* was reduced in *hs:sox2/hs:sox2* at 16.5 hpf but showed moderate expansion by 18.5 hpf (Fig. 5H, K). In *hs:sox3/hs:sox3* embryos heat shocked at 12.5 hpf, the domain of *atoh1a* was partially expanded at 16.5 hpf and continued to expand through 18.5 hpf (Fig. 5C, F). High-level misexpression of *sox3* did not accelerate the onset of *neurog1* expression, but the *neurog1* domain was already partially expanded by 16.5 hpf and continued to expand through 18.5 hpf (Fig. 5I, L). To test whether expansion of sensory and neurogenic domains involved elevated proliferation, we examined patterns of phospho-histone H3 staining after misexpressing *sox2* or *sox3* at 12.5 hpf. There was no significant change in the number of phospho-Histone H3 positive cells in the otic vesicle in *hs:sox2/hs:sox2* or *hs:sox3/hs:sox3* embryos at any point before or during sensory-neural expansion (Fig. 5M, N). Thus, early activation of *sox2* or *sox3* did not immediately or directly induce sensory and neural fates, nor promote cell proliferation to expand the sensory and neurogenic domains. Instead, the delayed response to misexpression suggests that transient elevation of *sox2* or *sox3* during placodal stages causes lasting changes in developmental programming that enhance competence to form sensory and neural fates in the otic vesicle.

Effects of misexpression of *sox2* and *sox3* on patterning in the otic vesicle

The strong expansion of sensory and neurogenic domains following high-level misexpression of *sox2* or *sox3* suggested dramatic changes in patterning of the otic vesicle. To test this, we analyzed the expression of a variety of regional markers at 24 hpf after high-level misexpression of *sox2* or *sox3* at 12.5 hpf. Anterior markers *fgf3* and *pax5* were expanded throughout the medial wall of the otic vesicle at 24 hpf, as was *fgf8* (Fig. 6A-C''), and the anterior-ventral marker *hmx3a* expanded to include nearly all cells in otic vesicle (Fig. 6E-E''). Medial expression of *pax2a* was not altered (Fig. 6D-D'') but the posterior-medial marker *pou3f3b* was lost in all embryos (Fig. 6H-H''). Posterior-lateral markers *otx1b* and *gsc* and the dorsal marker *dlx3b* were strongly reduced in *hs:sox2/hs:sox2* embryos and were eliminated in *hs:sox3/hs:sox3* embryos (Fig. 6F-F'', G-G'', J-J''). Similarly, *tbx1*, a marker of non-neural and non-sensory fates, was nearly eliminated in all embryos (Fig. 6I-I''). These data show that early misexpression of *sox2* and *sox3* leads to expansion of anterior-ventral identity in almost all cells in the otic vesicle, corresponding to the region shared by utricular sensory and neural fates. Despite this dramatic change in axial patterning, most embryos maintained a clear sensory-neural boundary, corresponding to the lateral boundary of the *pax2a* domain (see below).

Mosaic misexpression of *sox3*

Because global misexpression could potentially alter surrounding tissues that normally provide signals needed for proper patterning of the otic vesicle, we transplanted *hs:sox3/hs:sox3* cells into wild-type host embryos to test the effects of mosaic misexpression. We reasoned that if early misexpression of *sox3* acts cell-autonomously to enhance sensory-neural competence, then transplanted *hs:sox3/hs:sox3* cells should be able to adopt sensory and neural fates in ectopic locations within the otic vesicle. In support, when mosaic embryos were heat shocked at 12.5 hpf, 26.7% of transplanted *hs:sox3/hs:sox3* cells located in regions outside endogenous sensory epithelia expressed *atoh1a* ectopically at 24 hpf (n=32 embryos, 105 transplanted cells) (Fig. 7A, B, E), whereas no wild-type cells transplanted into wild-type host embryos expressed *atoh1a* ectopically (n=8 embryos, 63 ectopic transplanted cells) (Fig. 7E). Similarly, 31.9% of transplanted *hs:sox3/hs:sox3* cells located outside the endogenous neurogenic domain expressed *neurog1* ectopically at 24 hpf (n=9 embryos, 72 transplanted cells) (Fig. 7C, D, F), whereas no control transplants expressed *neurog1* ectopically (n=11 embryos, 83 ectopic transplanted cells) (Fig. 7F). These data support the idea that elevating *sox3* at 12.5 hpf cell-autonomously enhances pro-sensory and pro-neural competence of otic cells.

Interaction with *pax2a* influences *sox2* and *sox3* function

Previous studies suggested that SoxB1 factors can physically or genetically interact with other transcription factors to modify their functions (Ambrosetti et al., 1997; Boer et al., 2007; Chew et al., 2005; Kamachi et al., 2001; Kondoh and Kamachi, 2010). Because *pax2a* expression is restricted to the medial wall of the otic vesicle and helps regulate sensory development (Riley et al., 1999), we hypothesized that Pax2a locally biases the activity of Sox2 and Sox3 to promote sensory fate. To test this, we examined the effects of misexpressing *sox2* or *sox3* in *pax2a*^{-/-} mutants or *pax2a* morphants. Disruption of *pax2a* function did not block formation of endogenous sensory epithelia (Fig. 8C; and Riley et al., 1999) but suppressed the ability of early high-level misexpression of *sox2* to expand the sensory domain of *atoh1a* at 24 hpf (compare Fig. 8B, D). Importantly, knocking down *pax2a* did not suppress the ability of *sox2* to expand the neurogenic domain of *neurog1* (Fig. 8F, H). Similarly, disruption of *pax2a* suppressed the ability of *sox3* misexpression to expand the sensory domain of *atoh1a* whereas expansion of the neurogenic domain of *neurog1* still occurred (data not shown). These data support the hypothesis that the pro-sensory effect of Sox2 and Sox3 requires Pax2a, whereas the pro-neural function does not.

Mutual repression between *atoh1a* and *neurog1*.

In mouse embryos, neurogenesis and sensory development occur sequentially from the same spatial domain. The transition from neurogenesis to sensory development is regulated in part by mutual repression between *Neurog1* and *Atoh1* (Raft et al., 2007). To test whether a similar cross-repression helps reinforce or maintain the sensory-neural boundary in zebrafish, we activated *hs:atoh1a*⁺ or *hs:neurog1*⁺ at 24 hpf when sensory and neurogenic domains are already well established and then examined subsequent effects on *neurog1* or *atoh1a* expression at 30 hpf. We previously reported that serial activation of *hs:atoh1a* by heat shocking embryos 24 hpf and 27 hpf provides optimal expansion of sensory epithelia

(Sweet et al., 2011). Under these same conditions, serial activation of *hs:atoh1a* eliminated expression of *neurog1* at 30 hpf (Fig. 9C, D). Conversely, serial activation of *hs:neurog1* at 24 hpf and 27 hpf strongly repressed expression of *atoh1a* at 30 hpf (Fig. 9A, B). These data support the idea that the mutual antagonism between *atoh1a* and *neurog1* helps maintain the sensory-neural boundary during otic development in zebrafish.

Because *Neurog1-Atoh1* cross-repression in mouse is mediated in part by Notch signaling (Raft et al., 2007), we examined whether disruption of Notch signaling in *mind bomb (mib)* mutants alters the sensory-neural boundary in zebrafish. Two-color in situ hybridization showed that *mib* mutants exhibit strong upregulation of both *atoh1a* and *neurog1*, but the *atoh1a-neurog1* boundary is nevertheless maintained (Fig. 9E, F). This indicates that Notch activity represses expression of both *atoh1a* and *neurog1* but is not required for spatial segregation of sensory and neural fates in zebrafish.

Early expansion of sensory potential by Fgf

The earliest sign of sensory development occurs at roughly 10.5 hpf (tail bud stage) when *atoh1b* is induced at the medial edge of the otic placode (Millimaki et al., 2007), less than one hour after Fgf-dependent induction of *pax8* in the nascent otic anlagen (Phillips et al., 2001). Expression of *atoh1b* is later required for timely activation of *sox2* in the sensory domain (Millimaki et al., 2010). We showed previously that maintaining expression of *atoh1b* requires Fgf (Millimaki et al., 2007), but this is difficult to interpret because blocking Fgf at 10 hpf destabilizes otic development (Léger and Brand, 2002). Additionally, high-level misexpression of Fgf at 10 hpf enlarges the entire otic placode (Padanad et al., 2012), including the early domain of *atoh1b* expression. We therefore tested whether misexpressing Fgf at a low level could expand the domain of *atoh1b* without increasing the number of *pax8*-expressing otic cells. For this experiment, *hs:fgf8*⁺ heterozygotes were subjected to a very mild heat shock at 35°C beginning at 10 hpf and embryos were fixed at 10.8hpf to examine expression of *atoh1b*. Under these conditions, the size of the *pax8* domain was not altered (Fig. 10A, B, E) but the domain of *atoh1b* expression expanded by 34% based on measuring spatial area or by counting *atoh1b*⁺ cells (Fig. 10C, D, F, G). The enlarged domain of *atoh1b* was not maintained after the heat shock, as the number of *atoh1b*⁺ cells declined to normal by 14 hpf (not shown) in a process previously shown to involve Notch-dependent domain-restriction (Millimaki et al., 2007). Nevertheless, these data indicate that the level of Fgf signaling can influence the proportion of pre-otic cells able to express prosensory markers.

DISCUSSION

Our findings support a model in which *sox2* and *sox3* provide unique functions during placodal development to establish sensory and neurogenic competence, respectively (Fig. 11A). Previous studies suggest that SoxB1 functions can vary based on intrinsic differences in protein structure, expression level, or availability of cofactors. It appears that all three variables influence *sox2* and *sox3* functions during otic development in zebrafish. First, several observations suggest that the functions of *sox2* and *sox3* are intrinsically different. Although expression of *sox2* and *sox3* overlap in the prosensory region, mutant analysis

shows that only *sox2* is required for normal sensory development, and double mutants show no further impairment. Additionally, moderate misexpression of *sox2* expands sensory domain while reducing the neurogenic domain. Second, the functions of both genes depend on their level of expression. Specifically, *sox2* and *sox3* can mimic each other when misexpressed at high levels, leading to dramatic expansion of the sensory and neurogenic domains in the otic vesicle (Fig. 11B). Third, regionally expressed cofactors appear to modify the functions of Sox2 and Sox3: The medial factor *pax2a* is required to expand the sensory domain following misexpression of either *sox2* or *sox3*, whereas the neurogenic domain is unaffected (Fig. 11A, B). Thus the overlap of multiple mechanisms allows otherwise similar SoxB1 factors to perform distinct functions in abutting domains of the otic vesicle.

The ability of *sox2* and *sox3* misexpression to dramatically expand sensory and neurogenic domains correlates with global expansion of the anterior-ventral markers of the otic vesicle (Fig. 6). This phenotype is remarkable in several ways. First, regional fates in the otic vesicle normally rely on inductive interactions from surrounding tissues. However, early misexpression of *sox2* or *sox3* stably specifies anterior-ventral identity. That is, cells are not respecified by signals that normally establish distinct regional identities. Although global misexpression of *sox2/3* potentially alters such signals, genetic mosaics containing isolated transgenic cells also show a high incidence of sensory or neural development in ectopic locations. With such sparse distribution of transgenic cells it is unlikely that signaling interactions from surrounding tissues are significantly altered, suggesting that sensory and neural competence persists regardless of changing regional signals. We speculate that *sox2* and *sox3* function in the otic vesicle much as they do in the early neural plate, wherein SoxB1 factors stably specify a zone of neurogenic potential while simultaneously preventing premature neural differentiation. Subsequently, rising levels of neurogenic bHLH transcription factors repress expression of SoxB1 factors as cells begin to differentiate. Such a transition is seen in the neurogenic domain of the otic vesicle, wherein *sox3* is expressed in a gradient with levels declining towards the lateral edge where neuroblasts delaminate (Fig. 1E; Kantarci et al., 2016). The transition is also evident in sensory epithelia when hair cells upregulate *atoh1a* while losing expression of *sox2* (Millimaki et al., 2010).

The second notable feature of the phenotype caused by early misexpression of *sox2* or *sox3* is that the medial-lateral segregation of sensory and neurogenic fates is maintained and continues to respect the *pax2a* expression boundary. Although *pax2a* expression is initially expressed throughout the otic placode, it overlaps with the medial/prosensory domain of *sox2* as early as 12 hpf (6 somites, Gou et al., 2017). As the otic vesicle forms, expression of *pax2a* becomes restricted to the medial wall but continues to overlap with *sox2* in the ventromedial quadrant. Expression of *pax2a* does not depend on *sox2* but is nevertheless required for expansion of sensory epithelia by *sox2/3* misexpression. Thus the *sox2-pax2a* partnership defines the prosensory compartment throughout early otic development. In contrast, the ability of *sox3* to promote neural competence appears to require the absence of *sox2* (Fig. 2G), whereas *pax2a* is superfluous. Neurogenic competence neither requires *pax2a* nor is it impaired by misexpression of *pax2a* (Kantarci et al., 2016).

Basis for unique functions of Sox2 and Sox3

Although most studies conclude that Sox2 and Sox3 functions are largely redundant, there are several examples in which these proteins exhibit distinct functions. For instance, human embryonic stem cells (hESCs) express both *Sox2* and *Sox3*, but their functions are not identical. Either factor is sufficient to maintain pluripotency, but *Sox2* alone can promote hESC self-renewal whereas *Sox3* cannot (Wang et al., 2012). In neural progenitors, too, some functions of *Sox2* and *Sox3* are non-redundant as each factor activates a different set of neural makers (Archer et al., 2011; Rogers et al., 2009; Rogers et al., 2014). The mechanistic basis for such differences is unknown but possibly reflects structural differences in the transactivation domain that alter the ability to interact with different cofactors (Cox et al., 2010; Kondoh and Kamachi, 2010). Additionally, Sox2 and Sox3 can show markedly different affinities for specific DNA sequences (Collignon et al., 1996), indicating that small changes in their HMG DNA-binding domains also facilitate distinct functions.

Comparison with chick and mouse

In chick, *Sox2* and *Sox3* show overlapping expression during otic development. Expression of *Sox3* begins during early placodal development, marking the nascent neurogenic domain and promoting subsequent expression of *Ngn1* (Abello et al., 2010). Expression of *Sox2* begins later and is retained in sensory epithelia, whereas *Sox3* expression is lost after the neural-sensory transition (Neves et al., 2007). These data support a functional bias for *Sox3* in neurogenic competence and *Sox2* for sensory competence, similar to what we found in zebrafish. Overexpression of *Sox2* in chick can induce *Ngn1* (Evsen et al., 2013), though under these conditions it may mimic the normal function of Sox3 as we have found in zebrafish.

In mouse, *Sox2* is expressed in the otic vesicle and is required for both neural and sensory development (Kiernan et al., 2005; Puligilla et al., 2010; Steevens et al., 2017), whereas *Sox3* expression is not detected. How *Sox2* alone mediates both functions is not understood, but comparison with zebrafish suggests several possibilities. First, the level of *Sox2* expression could be sufficiently high in mouse that it can fulfill both functions, similar to our misexpression studies in zebrafish. Second, functional output could be influenced by interactions with *Pax2*. The early neurogenic domain in mouse straddles the *Pax2* expression boundary (Burton et al., 2004), and it is likely that most neuroblasts arise from the lateral (*Pax2*-negative) domain. The lateral neurogenic domain overlaps a ventrolateral domain of *Goosecoid* (Vitelli et al., 2003), which in zebrafish is required for neuroblasts to delaminate and is repressed by *pax2a* (Kantarci et al., 2016). In mouse, vestibular and auditory neurons do not detectably express *Pax2* (Lawoko-Kerali et al., 2002), and loss of *Pax2* does not block formation of vestibular or spiral ganglia (Burton et al., 2004). In contrast, *Pax2* is abundantly expressed in sensory epithelia, especially in differentiating hair cells (Lawoko-Kerali et al., 2002), and loss of *Pax2* perturbs development of sensory epithelia (Burton et al., 2004; Zou et al., 2006). It is possible that Pax2 physically interacts with Sox2 to activate sensory-specific enhancers, analogous to Pax6-Sox2 activation of lens-specific enhancers (Kamachi et al., 2001). Alternatively, Pax2 and Sox2 can also bind independently to widely separated binding sites within specific enhancers to drive expression in sensory epithelia (Robert-Moreno et al., 2011).

Relative roles *Neurog1*-*Atoh1a* cross-repression and Notch

The transition from neural to sensory development in birds and mammals is triggered in part by *Neurog1*-dependent activation of Notch (Brooker et al., 2006; Daudet et al., 2007; Daudet and Lewis, 2005; Neves et al., 2011), as well as cross-repression between *Neurog1* and *Atoh1* (Raft et al., 2007). In zebrafish, too, *atoh1a* and *neurog1* show cross-repression which presumably helps maintain and sharpen the sensory-neural border. However, Notch activity plays no role in spatial segregation between these domains in zebrafish, since a sharp boundary persists in *mib* mutants. Rather Notch acts independently in both domains to limit *atoh1a* and *neurog1* activity. This function is critical for establishing the alternating pattern of hair cells and support cells in sensory epithelia (Haddon et al., 1998; Millimaki et al., 2007; Riley et al., 1999) and to limit the pace of neuroblast specification and differentiation (Kantarci et al., 2015).

The role of Fgf

The earliest sign of prosensory development in zebrafish is the induction of *atoh1b* in the medial portion of the otic placode (Millimaki et al., 2007). Activation of *atoh1b* requires Fgf signaling and is limited to medial cells in close proximity to the hindbrain-source of Fgf. We showed that low-level activation of *hs:fgf8* is sufficient to increase the number of *atoh1b*-expressing cells without increasing the total number of *pax8*-positive otic cells (Fig. 10). The early otic domain of *atoh1b* does not reflect overt sensory specification since the domain is later restricted to only a few prospective hair cells by Notch-dependent lateral inhibition (Millimaki et al., 2007). However, the early *atoh1b* domain can nevertheless be viewed as an early marker of prosensory competence or potential, since knockdown of *atoh1b* blocks differentiation of the first hair cells and delays expression of *atoh1a* by many hours (Millimaki et al., 2007; Millimaki et al., 2010). While the above data suggest that elevating Fgf expands prosensory competence, it is unclear whether there is a corresponding contraction of neurogenic competence. However, we showed previously that weak activation of *hs:fgf8* does cause downregulation of *sox3* to a discrete lower level in the otic placode (Bhat and Riley, 2011; Padanad and Riley, 2011). Whether this molecular change reflects reduced neurogenic competence remains to be determined.

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HIGHLIGHTS

- Sox2 uniquely promotes sensory competence while Sox3 promotes neural competence.
- Transient overexpression of Sox2/3 expands both sensory and neurogenic domains.
- Sensory-neural segregation involves Atoh1a-Neuorg1 cross-repression but not Notch.
- Sensory competence and Sox2 function are favored by Pax2a and elevated Fgf.

Figure 1. Expression of *sox3* in the otic vesicle overlaps sensory and neurogenic domains
 (A) Schematic depiction of the floor of the otic vesicle (anterior up, lateral to the left) showing expression domains of *atoh1a* in the utricular (ut) and saccular (sac) maculae, *neurog1* in the neurogenic domain, and the medial marker *pax2a*. Dashed lines indicate the section planes shown in (B-D) and (E-G). (B-D) Expression of *sox3* (B), *pax2a* (C) and *atoh1a* (D) in cross sections passing through the utricular macula at 24 hpf. (E-G) Expression of *sox3* (E), *pax2a* (F) and *neurog1* (G) in cross sections passing through the widest part of the neurogenic domain at 24 hpf. Otic vesicle borders are outlined in B-G.

Figure 2. Distinct roles for *sox2* and *sox3* in sensory and neural development

(A, B) Box-and-whisker plots of the total number of hair cells at 38 hpf (A) and mature SAG neurons at 36 hpf (B) in control, *sox2*^{-/-}, *sox3*^{-/-}, and *sox2*^{-/-}; *sox3*^{-/-} double mutant embryos. Green lines represent means. Asterisks indicate statistically significant differences compared to controls (****P*<0.001, Tukey's HSD test following ANOVA). (C-H) Dorsolateral views (anterior to left) of expression of *atoh1a* (C-E) and *neurog1* (F-H) at 24 hpf in control embryos, *hs:sox2*^{+/+} heterozygotes and *hs:sox3*^{+/+} heterozygotes. Embryos were heat shocked at 12.5 hpf, 38°C or 39°C for 30 minutes, as indicated. (I-L) Expression of *atoh1a* (I, J) and *neurog1* (K, L) at 24 hpf in cross sections through the middle of the otic vesicle in control embryos (I, K) and *hs:sox3*^{+/+} heterozygotes (J, L). Embryos were heat shocked at 12.5 hpf, 38°C for 30 minutes. Otic vesicle borders are outlined in C-L. (M, N) Quantification of the total number of hair cells (M) and mature SAG neurons (N) at 30 hpf in control and *hs:sox2*^{+/+} embryos. (O, P) Quantification of the total number of hair cells (O) and mature SAG neurons (P) at 31 hpf in control and *hs:sox3*^{+/+} embryos. Error bars represent standard deviation in M-P, and asterisks indicate statistically significant differences relative to controls (**P*<0.05, ****P*<0.001, student's *t*-test, *n*>13).

Figure 3. Effects of high-level misexpression of *sox2* or *sox3* during early placode development (A-H) Dorsolateral views (anterior to the left) of expression of *atoh1a* (A-D) and *neurog1* (E-H) at 24 hpf in control (A, E), *hs:sox2/hs:sox2* (B, F) and *hs:sox3/hs:sox3* (C-H) embryos. (I-N) Expression of *atoh1a* (I-K) and *neurog1* (L-N) at 24 hpf in cross sections (lateral to the left) through the middle of the otic vesicle in *hs:sox2/hs:sox2* homozygotes (I, L) and *hs:sox3/hs:sox3* homozygotes (J, K, M, N). *hs:sox2/hs:sox2* homozygotes were heat shocked at 12.5 hpf, 39°C for 60 minutes, whereas *hs:sox3/hs:sox3* homozygotes were heat shocked at 12.5 hpf, 38°C for 30 minutes. Otic vesicle borders are outlined.

Figure 4. Effects of misexpressing *sox2* or *sox3* at later stages

(A-H) Expression of *atoh1a* (A-D) and *neurog1* (E-H) at 24 hpf in control (A, E), *hs:sox3/+* (B, F), *hs:sox3/hs:sox3* (C, G) and *hs:sox2/hs:sox2* (D, H) embryos. Embryos were heat-shocked at 18 hpf with varying temperatures and durations as indicated. White arrows indicate otic expression of *neurog1* in G. (I-N) Expression of *atoh1a* at 26 hpf (I-K) and 30 hpf (L-N) in control (I, L), *hs:sox2/hs:sox2* (J, M) and *hs:sox3/hs:sox3* (K, N) embryos following heat-shock (39°C for 60 minutes) at 24 hpf. Black arrowhead indicates *atoh1a* expression in the saccular macula in N, which is strongly reduced compare to L. (O-T) Expression of *neurog1* at 26 hpf (O-Q) and 30 hpf (R-T) in control (O, R), *hs:sox2/hs:sox2* (P, S) and *hs:sox3/hs:sox3* (Q, T) embryos following heat-shock (39°C, 60 minutes) at 24 hpf. All images show dorsolateral views (anterior to the left) and otic vesicle borders are outlined.

Figure 5. *sox2* and *sox3* do not directly specify neural or sensory fates

(A-F) Dorsolateral views (anterior to the left) showing otic expression of *atoh1a* at 16.5 hpf (A-C) and 18.5 hpf (D-F) in control (A, D), *hs:sox2/hs:sox2* (B, E) and *hs:sox3/hs:sox3* (C, F) embryos following heat-shock at 12.5 hpf. (G-L) Dorsolateral views showing otic expression of *neurog1* at 16.5 hpf (G-I) and 18.5 hpf (J-L) in control (G, J), *hs:sox2/hs:sox2* (H, K) and *hs:sox3/hs:sox3* (I, L) embryos following heat-shock at 12.5 hpf. Otic vesicle borders are outlined in all images. (M, N) Quantification of the number of phospho-Histone H3 positive (PH3+) cells in the otic vesicle of control and *hs:sox2/hs:sox2* (M) or *hs:sox3/hs:sox3* (N) embryos at multiple time points following heat-shock at 12.5 hpf. Error bars represent standard deviation. No statistically significant differences between control and *hs:sox2/hs:sox2* or *hs:sox3/hs:sox3* embryos at any time point examined (student's t-test, $n > 15$). In all panels, *hs:sox2/hs:sox2* embryos were heat shocked at 39°C for 60 minutes, whereas *hs:sox3/hs:sox3* embryos were heat shocked at 38°C for 30 minutes.

Figure 6. Axial patterning in the otic vesicle following early high-level misexpression of *sox2* or *sox3*.

Dorsolateral views (anterior to the left) showing expression of various regional markers at 24 hpf in the otic vesicle of control (A-J), *hs:sox2/hs:sox2* (A'-J') and *hs:sox3/hs:sox3* (A''-J'') embryos following heat-shock at 12.5 hpf. *hs:sox2/hs:sox2* embryos were heat shocked at 39°C for 60 minutes, *hs:sox3/hs:sox3* embryos were heat shocked at 38°C for 30 minutes. Otic vesicle borders are outlined in all images.

Figure 7. Effects of early misexpression of *sox3* in genetic mosaics

(A-D) Expression of *atoh1a* (A) and *neurog1* (C) at 24 hpf in cross sections of wild-type hosts into which fluorescent dextran-labeled *hs:sox3/hs:sox3* transgenic cells were transplanted. Mosaic embryos were heat shocked at 12.5 hpf, 38°C for 30 minutes. (B, D) Fluorescent image of dextran and DAPI staining on the same sample shown in A and C respectively. Sections pass through the middle of the otic vesicle, just posterior to the utricular macula. Arrows indicate transgenic cells that ectopically express *atoh1a* or *neurog1*. Otic vesicle borders are outlined. (E, F) Quantification of the percentage of transgenic cells located outside endogenous sensory or neural domains that ectopically express *atoh1a* (E) or *neurog1* (F).

Figure 8. Pax2a is required for prosensory but not proneural expansion

(A-H) Dorsolateral views (anterior to the left) showing otic expression of *atoh1a* (A-D) and *neurog1* (E-H) at 24 hpf in control embryos (A, E), *pax2a*-morphants (C, G), *hs:sox2/hs:sox2* homozygotes (B, F) and *hs:sox2/hs:sox2* homozygotes injected with *pax2a*-mo (D, -H). Embryos were heat shocked at 12.5 hpf, 39°C for 60 minutes. Otic vesicle borders are outlined in all images.

Figure 9. Roles for *atoh1a*-*neurog1* cross-repression but not Notch in sensory-neural segregation
 (A, B) Expression of *atoh1a* at 30 hpf in a control (A) and *hs:neurog1/+* (B) embryo following serial heat shock at 24 and 27 hpf, for 60 min at 39°C. (C, D) Expression of *neurog1* at 30 hpf in a control (C) and *hs:atoh1a/+* (D) embryo following serial heat shock at 24 and 27 hpf, for 60 min at 39°C. (E, F) Co-staining of *atoh1a* (red) and *neurog1* (black) expression by two-color in situ hybridization in a control embryo (E) and a *mib* homozygote mutant (F) at 24 hpf. Otic vesicle borders are outlined in all images. The width of the utricular macula is marked by vertical lines. All images show dorsolateral views with anterior to the left.

Figure 10. Low-level misexpression of Fgf8 expands sensory potential in the early placode
 (A-D) Dorsal views showing expression of *pax8* (A, B, anterior up) and *atoh1b* (C, D, anterior to the left) at 10.8 hpf in control (A, C) and *hs:fgf8/+* (B, D) embryos that were heat shocked at 10 hpf, 35°C for 45 minutes. (E-G) Quantification of relative surface area of otic/epibranchial *pax8* domain (E), *atoh1b* domain (F) and the number of *atoh1b*-expressing cells (G) in control and *hs:fgf8/+* embryos following heat shock at 10 hpf, 35°C for 45 minutes. Error bars represent standard error of the mean. Asterisks indicate statistically significant differences compare to control (* $P < 0.05$, student's t-test, $n > 22$).

Figure 11. Summary and model of sensory-neural patterning

(A) Under normal conditions, a gradient of Fgf from the mesendoderm and hindbrain induces the formation of otic placode, in which *pax2a* and *sox3* are uniformly expressed. By 12 hpf, however, *sox3* expression is reduced in medial cells and *sox2* is restricted to medial cells, changes that reflect ongoing Fgf signaling. Later, expression of *pax2a* becomes limited to the medial wall of the otic vesicle. Sensory competence is favored by the overlap between *sox2*, *pax2a*, elevated Fgf, and reduced *sox3*. Neurogenic competence is favored by elevated *sox3*, the absence of *sox2*, and moderate Fgf. These early patterns help establish spatial segregation of sensory and neurogenic domains in the floor of the otic vesicle (with the anterior half represented here), in which cross-repression between Neurog1 and Atoh1a helps reinforce or maintain segregation. (B) When Sox2/3 is transiently overexpressed at 12.5 hpf, prosensory and proneural competence increases in all otic cells. Later, sensory and neurogenic domains expand throughout the otic vesicle. This is accompanied by loss of non-sensory and non-neural fates and expansion of anterior-ventral identity throughout the otic vesicle, the region normally shared by utricular sensory and neural fates. Despite these changes in axial patterning, medial-lateral segregation of sensory and neural domains, and the medial domain of Pax2a, are maintained.